

1 Combining Ceftriaxone with Doxycycline and Daptomycin Reduces Mortality,
2 Neuroinflammation, Brain Damage and Hearing Loss in Infant Rat Pneumococcal Meningitis

3

4 Running title: Triple Antibiotic Therapy in Pneumococcal Meningitis

5

6 Lukas Muri^{1,2}, Michael Perny¹, Jonas Zemp¹, Denis Grandgirard¹ and Stephen L. Leib^{1,#}

7

8 ¹ Neuroinfection Laboratory, Institute for Infectious Diseases, University of Bern, Friedbühlstrasse 51, 3001 Bern

9 ² Graduate School for Cellular and Biomedical Sciences (GCB), University of Bern, Freiestrasse 1, 3012 Bern

10 [#] Corresponding author: Stephen.leib@ifik.unibe.ch

11

12 Corresponding author:

13 Stephen L. Leib
14 stephen.leib@ifik.unibe.ch
15 Institute for Infectious Diseases
16 University of Bern
17 Friedbühlstrasse 51
18 3001 Bern
19 Switzerland

20

21 E-mail addresses:

22 lukas.muri@ifik.unibe.ch
23 michael_perny@yahoo.de
24 jonas.1313@hotmail.com
25 denis.grandgirard@ifik.unibe.ch
26 stephen.leib@ifik.unibe.ch

Abstract

Despite available antibiotic therapy, pneumococcal meningitis (PM) is associated with a case fatality rate of up to 30% in high-income countries. Survivors often suffer from severe lifelong disabilities. An excessive inflammatory reaction drives the pathophysiology leading to brain damage and neurologic sequelae. We aimed to improve the outcome of experimental PM by simultaneously targeting different pathophysiological mechanisms with combined adjunctive therapies previously shown to be neuroprotective. *In vitro*, the anti-inflammatory effect of doxycycline and daptomycin were evaluated on primary rat astroglial cells stimulated with *S. pneumoniae*. Eleven day old infant Wistar rats were infected intracisternally with *S. pneumoniae* and randomized for treatment with ceftriaxone or combination adjuvant therapy consisting of ceftriaxone, daptomycin and doxycycline. During acute PM, combined adjuvant therapy with ceftriaxone, daptomycin and doxycycline increased survival rate from 64.1% to 85.8% ($p<0.01$) and alleviated weight loss compared to ceftriaxone monotherapy ($p<0.01$). Levels of inflammatory cytokines were significantly reduced by combined adjuvant therapy *in vitro* ($p<0.0001$) and in cerebrospinal fluid *in vivo* ($p<0.05$). In infected animals treated with combined adjunctive therapy, cortical damage was significantly reduced ($p<0.05$) and they showed a trend towards better hearing capacity three weeks after the infection ($p=0.089$), an effect which was significant in mildly infected animals (48dB vs 67.22dB, $p<0.05$). These mildly infected animals showed significantly reduced cochlear fibrous occlusion ($p<0.01$). By combining non-bacteriolytic daptomycin and anti-inflammatory doxycycline with ceftriaxone, their previously reported beneficial effects were cumulated and identified the triple antibiotic therapy as a promising therapeutic option for paediatric PM.

Key words

Pneumococcal meningitis, brain injury, neurologic sequelae, neuroinflammation, hearing loss, combination adjuvant therapy

52 Introduction

53 Acute bacterial meningitis is a severe illness with high mortality and morbidity – especially when
54 acquired during infancy or childhood – causing long-lasting neurofunctional deficits (e.g. hearing loss,
55 epilepsy, cerebral palsy and cognitive deficits), which tremendously influence quality of life in
56 affected children (1–6). Currently, *Neisseria meningitidis* and *Streptococcus pneumoniae* are the most
57 prevalent etiological agents for childhood meningitis beyond the neonatal age, as *Haemophilus*
58 *influenza* type b has been nearly eradicated since vaccine introduction (7). In high-income countries,
59 meningitis caused by *S. pneumoniae* and *N. meningitidis* presents case-fatality rates of 30% and 7%,
60 respectively (6, 8). Fatality rates are reported to be as high as 50% in resource-poor setting (9). The
61 risk for neurologic sequelae is especially high after pneumococcal meningitis (PM) (3), which causes a
62 massive infection of the central nervous system (CNS) with associated cortical necrosis and apoptosis
63 of dentate gyrus granular cells progenitors in the hippocampus, as found in human patients (10) and
64 animal models (11–14). Neural cell death is caused by multiple factors including bacterial toxins and
65 an excessive inflammatory reaction from the host (15–17). Together with the recruited neutrophils,
66 activated brain-resident microglia are able to produce large quantities of inflammatory cytokines,
67 reactive oxygen and nitrogen species (ROS and RNS), helping to eradicate the pathogen but also
68 contributing to the development of neuronal damage (15, 18, 19). Pathological cell death in the
69 hippocampus during acute PM correlates with learning and memory deficits (20–23). Furthermore,
70 PM induces damage in the peripheral nervous system (PNS) characterised by sensorineural hearing
71 loss caused by damage to hairs cells and spiral ganglion neurons in the inner ear (24–26), provoking
72 hearing impairments in up to 30% of survivors (3, 4, 6).

73 Clinical guidelines recommend the use of adjunctive dexamethasone – an anti-inflammatory
74 corticosteroid – for adult PM in high income countries (27, 28). However, adjuvant dexamethasone
75 failed to provide a beneficial effect on PM-induced mortality and hearing loss in children (27, 29) and
76 even aggravated mortality, acute brain injury and long-term learning deficits in different experimental
77 models of bacterial meningitis (23, 30, 31). Over the last few decades, alternative adjuvant therapies
78 including antioxidants, complement inhibitors, non-bacteriolytic antibiotics or matrix

79 metalloproteinase (MMP) inhibitors, which target different pathophysiological mechanisms during
80 acute PM were tested and have shown promising results in PM animal models (32–39). These
81 therapies were mostly evaluated as single adjuvant therapies or in combination with dexamethasone
82 (40, 41), being less relevant in paediatric meningitis. We recently postulated to combine successful
83 single adjuvants to more effectively reduce CNS and PNS damage thereby improving long-term
84 outcome by reducing neurologic sequelae after paediatric PM. This strategy was successfully tested in
85 the same experimental model as described in this present study by combining daptomycin (DAP) and
86 the matrix metalloproteinase inhibitor Trocade (42). An independent experimental study also reported
87 beneficial effects of combining adjuvant therapies to improve acute and neurofunctional outcomes
88 after murine PM (43).

89 DAP has previously been shown to clear pneumococci from CSF more rapidly than ceftriaxone (CRO)
90 without inducing bacterial lysis, thereby lowering the overall inflammatory burden in animal models
91 (44, 45). DAP penetrates into the CSF – especially after neurological infection (46, 47) – and reaches
92 5–11.5% of serum concentration mediating bactericidal effects (46–48). In infant rat PM, adjunctive
93 daptomycin reduced neuroinflammatory cytokines in the cerebrospinal fluid (CSF) and decreased
94 brain injury and hearing loss (32, 42). Apart from its antimicrobial activity, doxycycline (DOX) –
95 which is known to penetrate well into the brain and CSF (49) – also has multiple anti-inflammatory
96 effects by reducing cytokine release and inhibiting MMP activity (50–56). Adjuvant DOX reduced
97 mortality and injury to the brain and cochlea in experimental infant rat PM (56), similarly to other
98 MMP inhibitors that were shown to reduce blood-brain barrier permeability, inflammatory cytokines
99 in CSF, brain injury and mortality during acute bacterial meningitis (34, 42, 57, 58).

100 By combining adjunctive DAP and DOX therapies, we intend to target multiple pathophysiological
101 mechanisms responsible for brain injury during acute bacterial meningitis with the aim to integrate the
102 beneficial effects of both substances and improve the neurofunctional outcome after paediatric PM.

103

104 **Results**

105 **Antibiotic susceptibility of *S. pneumoniae* serotype 3**

106 The MIC of ceftriaxone for the used *S. pneumoniae* serotype 3 was determined to be 0.003mg/L.
107 MICs of daptomycin and doxycycline were both at 0.064mg/L. Determined MICs revealed
108 susceptibility of our *S. pneumoniae* serotype 3 strain to assessed antibiotics according to published
109 data (59, 60).

110 **Reduced release of inflammatory cytokines and nitric oxide *in vitro***

111 Inflammatory cytokines and nitric oxide (NO) were measured in astroglial cell culture supernatant 24
112 hours after concomitant application of living *S. pneumoniae* and antibiotics. Antibacterial but
113 bacteriolytic therapy with CRO induced a strong release of inflammatory cytokines (IL-1 β , IL-6, IL-
114 10 and TNF- α , Fig. 1 A-D) and NO (Fig. 1 E). After adjustment for multiple testing, DOX
115 monotherapy significantly reduced the release of IL-1 β (p<0.002), IL-6 (p<0.002), TNF- α (p<0.0001)
116 and NO (p<0.001) compared to CRO monotherapy. Accordingly, CRO+DOX significantly decreased
117 IL-6 (p<0.02), TNF- α (p<0.0001) and NO (p<0.001) levels. DAP monotherapy and combination
118 therapy with CRO+DAP reduced the inflammatory reaction to a greater extent with clearly lowered
119 inflammatory cytokines – except IL-10 – (all p<0.0001 for CRO vs. DAP and CRO vs. CRO+DAP)
120 and NO release (p<0.0001 for all combinations containing DAP). Notably, combined triple therapy
121 with CRO+DOX+DAP significantly decreased the release of IL-1 β , IL-6, TNF- α and NO (all
122 p<0.0001 for CRO+DOX+DAP vs. CRO) and was the only therapy to significantly reduce IL-10
123 concentrations compared to CRO monotherapy (p<0.03). *S. pneumoniae* exposure did not induce a
124 visible cytopathic effect on astroglial cells in any of the treatment groups, as determined by the
125 observation of intact monolayers (data not shown). Therefore, the reported reduction of NO and
126 cytokine production upon DOX and/or DAP therapy cannot be associated to an increased cell death
127 within these groups.

128 **Improved survival and increased weight gain in infant rats with PM**

129 A total of 156 infant Wistar rats were enrolled for this study. One animal was excluded after
130 unsuccessful infection with no detectable bacterial CSF titer at 18 hpi. All other infected animals were

131 included after having fulfilled at least one criterion for successful infection (positive bacterial CSF
132 titer $> 10^6$ cfu/ml, reduced clinical score, weight loss, changes in posture).

133 Infected rats showed reduced survival compared to uninfected controls (Fig. 2 A). Animals receiving
134 the combination adjuvant therapy, demonstrated a significant better survival compared to infected rats
135 with CRO monotherapy (85.8 % vs. 64.1%, long-rank $p=0.0052$). Compared to uninfected controls,
136 which constantly gained weight within 42 hours after mock-infection, animals with PM showed only a
137 slight weight increase within the first 18 hours (Fig. 2 B). Thereafter, infected animals treated with
138 CRO monotherapy lost weight (2.81 % weight loss at 42 hpi). This weight loss was reduced with
139 combined adjuvant therapy (0.8% weight gain at 42 hpi), resulting in a significantly higher relative
140 weight gain compared to infected animals with CRO monotherapy ($p=0.0022$). At all measured time
141 points, uninfected animals gained significantly more weight than their infected counterparts,
142 independent of treatment modality ($p<0.0001$). Upon infection, clinical scores were reduced and
143 reached a minimum at 24 hpi, whereas uninfected animals did not show any changes in clinical scores
144 at any time point (Fig. 2 C). Clinical scores of infected animals treated with combination adjuvant
145 therapy showed a trend to be higher compared to infected animals receiving CRO monotherapy at 24
146 hpi ($p=0.0884$). From 24 hpi on, infected rats started to recover and showed an improvement in
147 clinical scores at 42 hpi, still being significantly lower than those of uninfected control animals
148 ($p=0.0015$ for CRO and $p=0.0053$ for CRO+DOX+DAP).

149 **Reduction of inflammatory parameters in CSF *in vivo***

150 Inflammatory cytokines were measured in the CSF of infected animals before and at 6 and 24 hours
151 after treatment initiation (representing 18, 24 and 42 hpi). Before treatment, cytokine levels were
152 comparable between the two infected groups. Compared to animals treated with CRO monotherapy,
153 IL-1 β and IL-10 CSF levels were significantly reduced in rats receiving the combination adjuvant
154 therapy six hours after treatment initiation ($p=0.0004$ and $p=0.0128$, Fig. 3 A and C). IL-6 and TNF- α
155 revealed a trend towards reduced CSF levels ($p=0.071$ and $p=0.076$, Fig. 3 B and D). Twenty-four
156 hours after therapy start, IL-6 was significantly lower in animals receiving combined adjuvant
157 interventions ($p=0.0139$, Fig. 3 B). IFN- γ levels were similar between the two groups at all time points

(data not shown). Before therapy initiation, bacterial CSF titers were not different with $1.09 \pm 0.71 \times 10^8$ cfu/ml in the CRO group and $1.15 \pm 1.26 \times 10^8$ cfu/ml in the CRO+DOX+DAP group ($p=0.772$). Six hours after therapy begin, all animals treated with the combined adjuvant intervention had undetectable bacterial titers ($<10^2 \pm 0$ cfu/ml), whereas half of the animals receiving CRO monotherapy still showed detectable but very low bacterial titers in CSF ($3.06 \pm 0.89 \times 10^2$ cfu/ml), representing a significantly faster bacterial clearance in CSF with combined adjuvant therapy ($p=0.0236$, Fig. 3 E).

Reduced cerebral complication *in vivo*

PM-induced cerebral damage was assessed in all animals sacrificed at 42 hpi. Cortical necrosis was only found in animals with PM. Its extent was significantly higher in the group treated with CRO monotherapy compared to animals receiving combined adjuvant therapy (0.73 % [IQR 0-9.7, $n=15$] vs. 0% [IQR 0-0, $n=15$] $p=0.0302$, Fig. 3 F). Elevated levels of hippocampal apoptosis were only found in infected animals (data not shown). Here, however, no differences between the two treatment groups were found (CRO monotherapy: 0.89 ± 0.91 , $n=13$ vs. CRO+DOX+DAP therapy: 0.92 ± 1.26 , $n=15$ apoptotic cells per visual field, $p=0.943$).

Improvement of hearing capacity three weeks after infection

To investigate long-term neurologic sequelae induced by PM, hearing capacity was assessed by auditory evoked brainstem response (ABR) three weeks after infection. Representative ABR recordings from a control animal and an infected rat are shown in figure 4 A and B, respectively. Control animals showed detectable responses down to 35 dB, whereas the infected animal did not show any measurable signal below 65dB (Fig. 4 A and B). The average hearing threshold was 38.13 ± 2.39 dB or 38.75 ± 1.44 dB in uninfected animals that received CRO monotherapy or combined adjuvant therapy, respectively (Fig. 4 C). Compared to uninfected rats, infected animals showed significantly higher average hearing thresholds, irrespective of therapy regimen. Treatment with combined adjuvant therapy showed a trend for improved hearing capacity compared to CRO monotherapy (63.26 ± 23.9 dB vs. 72.88 ± 24.8 dB, $p=0.0891$, Fig. 4 C) in infected rats. In experiments with severely infected animals (defined as an in-litter mortality $> 15\%$), severe hearing

185 loss (average hearing threshold ≥ 80 dB) was observed, with no difference between therapies. When
186 considering only experiments with an in-litter mortality of less than 15% (mild infection experiments),
187 combination adjuvant therapy with DAP and DOX – compared to CRO monotherapy – significantly
188 reduced PM-induced hearing loss (48.00 ± 21.7 dB, $n=10$ vs. 67.22 ± 25.9 dB, $n=9$, $p=0.0482$).

189 Univariate linear regression (table 1) analysis revealed significantly increased hearing threshold with
190 increasing bacterial inoculum (70.5 dB per additional log of infection inoculum, $p<0.001$). Low in-
191 litter mortality and higher clinical score at 24 hpi were both found to predict attenuation of PM-
192 induced hearing impairments (-18.1 dB in case of mildly infected animals, $p=0.011$; -26.7 dB per
193 additional point of activity score at 24 hpi, $p<0.001$). Multivariate linear regression modelling (table 1)
194 – including infection inoculum, litter mortality and treatment regimen – displayed comparable
195 findings. After adjusting for in-litter mortality and bacterial inoculum, combination adjuvant therapy
196 was found to significantly reduce PM-induced hearing loss by 10.3 dB ($p=0.037$).

197 **Improved hearing is associated with reduced fibrous occlusion of the perilymphatic** 198 **space**

199 To investigate the reason for improved hearing capacity in mildly infected rats treated with
200 combination adjuvant therapy, histomorphological damage in inner ear was assessed by
201 immunofluorescence. Previous work showed that PM-induced hearing loss was accompanied by
202 reduced spiral ganglion neuron density in the cochlea – being a critical predictor for the efficacy of a
203 cochlear implant (25). Spiral ganglion neurons were quantified in the basal, middle and apical turn of
204 mildly-infected animals, as indicated in figure 5 A. No statistical differences were found between the
205 two treatment groups at any of the cochlear turns (fig. 5 C). In the present study, only few animals
206 showed a reduction in SGN density (< 2000 TUJ⁺ cells/mm², e.g. left middle turn in Fig. 5 A), as
207 compared to SGN density previously reported in mock-infected animals (25). Histomorphological
208 assessment of the cochlear turns revealed extensive fibrous occlusion of the perilymphatic space in
209 infected animals (Fig. 5 B). Fibrous occlusion was significantly reduced in infected animals treated
210 with the combined adjuvant intervention compared to animals receiving CRO monotherapy (Fig. 5 D).
211 In mildly-infected animals, a significant positive correlation was found for fibrous occlusion of the
212 perilymphatic space and hearing threshold (Fig. 5 E, $r=0.5051$, $p=0.0274$).

Discussion

Observational clinical studies repetitively found neurologic sequelae in patients surviving bacterial meningitis, with especially high risk after PM (3). A profound inflammatory reaction in the CNS and PNS is associated with cortical necrosis, hippocampal apoptosis and cochlear damage (11–13, 24–26). In addition to bacterial toxins, the host's excessive inflammatory reaction contributes to the observed neural cell death (15–17). Bacterial autolysis and also bactericidal antibiotics with antibiotic-dependent lysis of bacteria provoke a release of highly immunogenic products, promoting inflammatory processes and disease severity (15, 61–64). During PM, secreted MMPs from activated immune cells are crucially upregulated and associated with brain injury (15, 34, 65) with higher MMP-9 level being associated with the development of hearing impairment or secondary epilepsy in infected children (65). By degrading basal lamina components and tight junction proteins, MMP-9 weakens the blood-brain-barrier (BBB) (58, 66–68) and facilitates leukocyte extravasation and BBB leakage (33, 69). Additionally, MMPs contribute to the inflammatory reaction and brain injury via their sheddase and convertase activity, being able to cleave and activate inflammatory cytokines and chemokines (33, 70–72).

DOX has multiple anti-inflammatory effects by reducing cytokine release and inhibiting MMP activity (50–56). Pharmacologic inhibition of MMPs during acute bacterial meningitis resulted in the reduction of blood-brain barrier permeability, inflammatory cytokine levels in CSF, brain injury and mortality (34, 42, 57, 58). Selective MMP inhibitors (e.g. BB-94, BB-1101, GM-6001 or TNF-484) have not yet been successfully tested in clinical studies. DOX, on the other hand, has a well-characterized clinical safety profile (56). The herein used dosage of DOX was previously shown by others to suppress cerebral MMP-9 activity and reduce ischemic brain damage in rodents (73, 74). In experimental infant rat PM, adjuvant DOX reduced mortality and injury to the brain and cochlea (56).

DAP, a non-bacteriolytic but bactericidal antibiotic, integrates itself into the bacterial cell membrane, induces its depolarisation and biosynthesis inhibition (75–77). In previous work, we demonstrated a faster bacterial clearance from CSF with DAP leading to reduced inflammatory parameters and decreased cortical complication compared to standard CRO therapy in infant rat PM (45, 78). In the

240 same infant rat PM model, adjunctive DAP to CRO reduced PM-associated cerebral damage,
241 inflammatory cytokine levels in the CSF and improved hearing capacity (32, 42). In adult rats with
242 PM, DAP treatment attenuated cognitive impairment in surviving rats compared to CRO (79).

243 Our previous work on the herein reported experimental model – but also other independent
244 experimental work – confirmed the potential of combined adjuvant interventions targeting multiple
245 pathophysiological mechanisms during PM, thereby improving acute and neurofunctional outcomes
246 (42, 43). With this study, we were able to show that combined adjuvant therapies in experimental
247 paediatric PM cumulate previously reported beneficial effects of single adjuvants and significantly
248 improve acute neuropathology and neuroinflammation, thereby improving neurofunctional outcome
249 compared to standard CRO monotherapy. Combined adjuvant therapy with DAP and DOX was able to
250 improve survival and weight gain in infant rats infected with *S. pneumoniae* (Fig. 2 A, B). Adjuvant
251 DOX – but not DAP – previously demonstrated its capacity to significantly improve survival among
252 infant rats with PM (32, 56). Tetracyclines also demonstrated mortality reduction in experimental
253 sepsis models (80). On the other hand, adjuvant DAP was beforehand shown to improve weight
254 change after treatment initiation (32). Herein, adjuvant combination therapy with DOX and DAP
255 significantly reduced inflammatory cytokines in CSF *in vitro* and *in vivo* (Fig. 1 and 3). Despite
256 previous findings, which showed reduced CSF levels of TNF- α upon DOX-induced TACE inhibition
257 (56) and reduced CSF IL-6 levels with adjuvant DAP (32), we only found statistical trends toward
258 lower CSF levels of these cytokines six hours after treatment initiation (Fig. 3 B,D). Of note, animals
259 treated with combined adjuvant therapy showed slightly higher initial TNF- α and IL-6 levels (18 hpi)
260 with faster subsequent reduction after therapy initiation. In other experimental inflammatory diseases,
261 DOX was shown to inhibit interleukin-converting enzyme, thereby lowering the bioavailability of IL-
262 1 β (56, 81). In accordance with this data, we found significantly reduced IL-1 β levels six hours after
263 initiating therapy *in vivo* with combined adjuvant intervention compared to CRO monotherapy.
264 Therapy initiation with CRO monotherapy caused increased CSF levels of IL-1 β and IL-6, indicating
265 that bacterial burst by lytic antibiotic further aggravates cerebral inflammation (44, 45, 63), which we
266 were able to inhibit with adjuvant non-bacteriolytic DAP *in vitro* and *in vivo*. Our *in vitro* model of
267 neuroinfection and neuroinflammation with primary rat astroglial cells supported the concept of

268 induced neuroinflammation upon therapy initiation with a bacteriolytic antibiotic with clearly reduced
269 inflammatory cytokines and NO release by treatment with non-bacteriolytic antibiotics (Fig. 1). The
270 reduced induction of neuroinflammation *in vitro* by simultaneous application of CRO+DAP (and
271 CRO+DOX+DAP) underlines previous findings showing fast antibacterial action of DAP (45) and
272 thereby supports the concept of DAP's anti-inflammatory actions even when applied concomitantly
273 with CRO.

274 Animals receiving combined CRO+DOX+DAP showed significantly reduced cortical damage 42 hpi
275 (Fig. 3 F), without showing a better outcome in terms of hippocampal apoptosis (not shown). The
276 reduced cortical complication might be attributed to the overall reduction of CSF inflammation, as
277 previously found by single adjuvant DAP and DOX therapy (32, 56).

278 Combined adjuvant therapy with CRO+DOX+DAP improved hearing thresholds in mildly infected
279 infant rats (Fig. 4 D), whereas severely infected animals showed profound hearing loss (average
280 hearing threshold ≥ 80 dB), which might be beyond potential protection. These data were confirmed
281 by a multivariate linear regression showing that after adjusting for infection inoculum and high in-
282 litter mortality, rats with combined adjuvant therapy revealed improved hearing capacity by 10.3 dB
283 (Table 1). Histologic analysis revealed that spiral ganglion neuron density was not significantly
284 affected in mildly infected rats (Fig. 5 C) compared to previously published data of mock-infected
285 infant rats (25). Improved hearing capacity in animals with combined adjuvant therapy was correlated
286 to a significant reduction of fibrous occlusion of the perilymphatic space (Fig. 5 D, E). Fibrous
287 obliteration of the perilymphatic space after experimental PM has been described in a mouse model of
288 PM and positively correlated with increased hearing loss (37). During acute infection, leukocytes enter
289 the perilymphatic space contributing to the inflammatory processes (26). Resolution of the
290 granulocytic inflammation is expected to cause occlusion of the perilymphatic space with connective
291 tissue, potentially leading to cochlear ossification (26). As cochlear ossification can limit the access
292 for cochlear implantations (82), a reduction in fibrous obliteration with associated cochlear
293 ossification has important clinical consequences. Of note, cochlear hair cells and presynaptic ribbons
294 of surviving inner hair cells were not analysed during this study. The detected improvement in hearing

295 capacity with combined adjuvant DAP and DOX might also be attributed to a protection of cochlear
296 hair cells or their connectivity to the SGNs. In previous work, we demonstrated that connectivity of
297 inner hair cells to SGNs was already reduced after mild infection, whereas the inner hair cells
298 themselves remained unaffected (25). In mildly infected animals with low level fibrotic occlusion, loss
299 of inner hair cell connectivity might explain elevated hearing thresholds (Fig. 5 E).

300 In a comprehensive meta-analysis, adjunctive anti-inflammatory corticosteroids were shown to
301 improve the outcome of adults with PM in high income countries and children with meningitis caused
302 by *Haemophilus influenzae* type b, without showing a clear benefit of corticosteroids in children with
303 PM (27). Multiple experimental models of bacterial meningitis with adjunctive dexamethasone
304 demonstrated aggravated mortality and acute hippocampal injury with subsequent learning and
305 memory deficits, especially in infant rodents (23, 30, 31, 83, 84). As there is no substantial evidence
306 for the use of dexamethasone in children with PM, we did not include it in our study focussing on
307 paediatric PM. Further limitations of this study include the lack of direct comparison to single
308 adjuvant therapies. As the respective single adjuvant therapies were already tested in our laboratory on
309 the same model of PM, we decided to not include these therapy groups but to compare to previously
310 reported data (32, 56). Additionally, we would like to mention the limitation of direct intracisternal
311 inoculation of pneumococci to induce PM, which does not represent the pathophysiologic route of
312 infection via the bloodstream (15). Yet, development of meningitis after intranasal inoculation with
313 bloodstream spread and hematogenous CNS infection is only obtained in a small proportion of the
314 infected animals – even after experimentally induced extracellular matrix degradation (85) – limiting
315 the use of this inoculation method for specific research on meningitis. Furthermore, animals in our
316 experiments were treated for 5 days and not 10-14 days as recommended for humans (28). Previous
317 experiments showed, however, that this treatment duration resulted in a complete bacterial clearance
318 with associated recovery of clinical scores and weight loss (25, 32, 42). Lastly, resulting CSF
319 daptomycin levels of 0.5mg/L in patients with neurological infections (47, 86) are just slightly above
320 the MIC for *S. pneumoniae* (60) and this may affect the beneficial effects seen in our animal model.”

321 **Conclusion**

322 Combination adjuvant therapy with non-bacteriolytic DAP and DOX with its MMP-inhibitory and
323 anti-inflammatory properties caused faster bacterial clearance and reduced inflammatory CSF cytokine
324 levels, known to be mediators of brain damage during acute PM. Previously reported beneficial effects
325 of these single adjuvants were merged together by combined intervention and improved survival,
326 weight loss, cerebral complication and neurologic sequelae such as hearing loss. Therefore, we
327 conclude that combining adjuvant DAP and DOX with CRO is a promising therapeutic option to
328 improve the outcome of PM.

329

330 **Material and Methods**

331 **Infecting organism**

332 A clinical isolate of *Streptococcus pneumoniae* (serotype 3) from a patient with bacterial meningitis
333 was cultured overnight in brain heart infusion (BHI) medium, diluted 10 fold in fresh, pre-warmed
334 BHI medium, and grown for 5 h to reach the logarithmic phase as reported earlier (32, 58). The
335 bacteria were centrifuged for 10 min at 3100 x g, washed twice and re-suspended in sterile, pyrogen-
336 free saline (NaCl 0.85%). Bacteria were further diluted to the desired density by measuring the optical
337 density at 570nm (OD₅₇₀). Inoculum accuracy was determined by serial dilutions and plating on
338 Columbia sheep blood agar (CSBA) plates.

339 **Antibiotic susceptibility testing**

340 For MIC determination, blood agar plates were inoculated with *S. pneumoniae* serotype 3 – the strain
341 used within this study. MICs of ceftriaxone (0.002-32mg/L; Liofilchem srl, Italy), doxycycline (0.016-
342 256 mg/L, bioMérieux, USA) and daptomycin (0.016-256 mg/L, bioMérieux, USA) were determined
343 by using antibiotic gradient strips, according to the manufacturer's protocol and as described before
344 (45).

345 **In vitro model of neuroinfection and neuroinflammation**

346 Primary rat astroglial cells, isolated from the cortex and hippocampus of infant rats on post-natal day 3
347 were kept in culture medium (DMEM [Sigma-Aldrich, Merck Switzerland] with 5% FCS [Biochrom,
348 Germany], GlutaMAX™ [ThermoFisher, Switzerland] and antibiotic-antimycotic [ThermoFisher,
349 Switzerland]) for 11 days at 37°C with 5% CO₂, as reported elsewhere (87, 88). For stimulation
350 assays, cells were seeded in poly-L-ornithine-coated 24-well plates and kept for additional 3 days.
351 Subsequently, cell culture medium was replaced with phenol red-free and pyruvate-free DMEM
352 (Gibco, ThermoFisher, Switzerland) with 5% FCS and GlutaMAX™. Cells were stimulated with 3 x
353 10⁷ CFU/mL *S. pneumoniae* in logarithmic growth phase. Directly after stimulation with living
354 bacteria, antibiotics were added to the cell culture medium (10µg/ml CRO [Rocephine, Roche]),
355 1µg/ml DAP [Cubicin, Cubist Pharmaceuticals], 3µg/ml DOX [doxycycline hyclate, Sigma],

reflecting CSF concentrations found in patients or experimental studies focusing on neuroinfections (46–49, 86, 89, 90). Nitric oxide production was measured using Griess reagent (Sigma-Aldrich, Merck Switzerland). Briefly, 100 μ L Griess reagent was mixed with 100 μ L cell culture medium in 96-well plates. Absorbance was measured at 550 nm with a microplate reader (THERMOmax, Molecular Devices, USA). Nitrite concentrations were calculated from a NaNO_2 standard curve.

361

362 **Infant rat model of pneumococcal meningitis**

All animal studies were approved by the Animal Care and Experimentation Committee of the Canton of Bern, Switzerland (license BE 129/14), and followed the Swiss national guidelines for the performance of animal experiments. A well-established infant rat model of PM was used for the experiments as previously described (32, 58). Eleven days old Wistar rats together with their dams were obtained from Charles River Laboratories (Sulzfeld, Germany). The dams were provided with tap water and pellet diet at libitum. Litters were kept in rooms at a controlled temperature of 22 ± 2 °C. During the acute phase of the disease, animals were housed in conventional cages in a room with natural light. For long-term experiments after bacterial curing, animals were transferred to individually ventilated cages (IVC) in a room with controlled 12-hour light/dark cycles. Intracisternal infections were performed by injection of 10 μ L saline containing $7.33 \pm 3.4 \times 10^5$ cfu/ml living *S. pneumoniae*. Mock-infected control animals received an equivalent volume of saline. Pneumococcal meningitis was confirmed 18 h post infection (hpi) by quantitative analysis of bacterial titers in CSF samples, when the animals developed symptomatic disease. For this, 5 μ L of CSF were collected by puncture of the cisterna magna, followed by serial dilution and cultivation on columbia sheep blood agar (CSBA) plates. The infected animals were randomized for treatment with CRO (100mg/kg, Rocephine, Roche, twice daily [b.i.d]) plus: a.) combination adjuvant therapy (n=72) consisting of DAP (10 $\text{mg} \cdot \text{kg}^{-1} \text{d}^{-1}$, s.c. in saline, single dose; Cubicin, Cubist Pharmaceuticals) plus DOX (30mg/kg, i.p, once daily, Sigma, combined with CRO), or b.) saline in the control group (n=72). Antibiotic therapy with CRO was started at 18 hpi in all animals. Therapies involving DAP were started by the application of DAP followed by a 15 min-delayed application (i.e. at 18:15 hpi) of other therapies. For long-term

383 experiments assessing neurofunctional outcomes, CRO and DOX therapies were continued until day 5
384 after infection. Mock-infected animals either received the combination adjuvant therapy (n=6) or the
385 CRO monotherapy with vehicles (n=6). All animals received the same amount of fluids during the
386 experiments.

387 The rats were weighted and examined clinically at 0, 18, 24 hpi, and before sacrificing at 42 hpi, as
388 previously described (58). Activity scores represent 1 = coma; 2 = does not turn upright; 3 = turns
389 upright within 30 s; 4 = minimal ambulatory activity, turns upright in < 5 s; and 5 = normal. Weight
390 was assessed relative to weight at time of infection (per cent increase). Investigators were blinded for
391 treatment modalities. Spontaneous mortality was documented. Punctures of the cisterna magna were
392 performed using a 30-gauge needle to obtain CSF samples at 18, 24, and 42 hpi. CSF samples not used
393 for bacterial titer determination were centrifuged ($16'000 \times g$ at 4 °C for 10 min), and the supernatants
394 were frozen at -80 °C until further use. Animals were sacrificed with an overdose of pentobarbital
395 (150 mg/kg b.w., i.p. Esconarkon, Streuli Pharma AG, Uznach, Switzerland) at 42 hpi and perfused
396 with ice-cold 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) before their brains were
397 removed and fixed in 4 % PFA for histological analysis.

398 **Histomorphometric assessment of cortical damage and hippocampal apoptosis**

399 Damage to cerebral structures was quantified as previously described in all animals sacrificed at 42 hpi
400 (33, 91, 92). Briefly, brains were fixed in 4 % PFA and cryopreserved in 18 % sucrose in PBS at 4 °C
401 overnight. Coronal brain cryosections (45 μ m thick) obtained by systematic uniform sampling were
402 stained for Nissl substance with cresyl violet. Cortical damage was defined as areas of decreased
403 neuronal density. Histological features of apoptosis were quantified in 48 systematic visual fields
404 spanning the hippocampus of both hemispheres. Histologic assessments were performed and evaluated
405 by a person blinded to treatment modalities.

406 **Quantitative analysis of cytokine levels in the CSF**

407 A panel of cytokines previously found to be upregulated in PM (15, 93) – i.e. IL-1 β , IL-6, TNF- α , IL-
408 10 and IFN- γ – was assessed using a magnetic multiplex assay (Rat Magnetic Luminex® Assay,

409 R&D Systems, Bio-Techne) and a Bio-Plex 200 station (Bio-Rad Laboratories) as previously
410 described (25, 33). Undiluted cell culture supernatant or five microliters of CSF harvested and
411 centrifuged at 18, 24 and 42 hpi were diluted to a final volume of 50 μ l using the provided assay
412 buffer. At least 50 beads were measured for each analyte. Calibration curves from recombinant
413 standards were calculated with Bio-Plex Manager software (version 4.1.1) using a five-parameter
414 logistic curve fitting. For samples below the detection limit, the value of detection limit provided by
415 the manufacturer (TNF- α , 22.1 pg/ml; IL-6, 56.0 pg/ml; IL-1 β , 26.7 pg/ml; IL-10, 18.6 pg/ml; IFN- γ ,
416 70.5 pg/ml) was multiplied by the dilution factor.

417 **Click- and pure tone-evoked auditory brainstem response**

418 Auditory brainstem responses (ABRs) were recorded in response to click stimulations and pure tones
419 on both ears using the SmartEP system (Intelligent Hearing Systems, Miami, USA), as previously
420 described (25). Animals were anesthetized with isoflurane (5 % for induction and 2 % for
421 maintenance) using the Combi-Vet Vaporizer System equipped with a digital flowmeter (Rothacher
422 Medical, Switzerland). 100 μ s click stimuli and five millisecond pure tone pips (Blackman envelope;
423 polarity alternating) were presented at a rate of 21.1 s⁻¹, ranging from 100 to 20 dB SPL in 10 dB
424 decrements (5 dB decrements close to threshold). A total of 1024 responses were averaged at each
425 sound level and filtered between 100 and 1500 Hz. The hearing threshold was defined as the lowest
426 intensity that induced the appearance of a visually detectable first peak. ABRs were recorded between
427 P30 to P34. Hearing thresholds were independently analysed and discussed by multiple blinded
428 investigators.

429 **Immunohistological analysis of spiral ganglion neuron density and cochlear occlusion**

430 Immunohistological analysis of the cochlea was performed as reported earlier (25). In summary, three
431 weeks after infection, the animals were sacrificed and perfused with 4% paraformaldehyde (PFA).
432 Cochleas were dissected and isolated followed by overnight fixation in 4% PFA at 4°C. Samples were
433 decalcified with Osteosoft (Merck) for 10 d before dehydration and cryopreservation in 30% sucrose
434 followed by cryosectioning. Fourteen μ m midmodiolar sections were cut and mounted on Superfrost

435 Plus microscopy slides (Thermo Fisher Scientific). The immunofluorescence procedure was
436 performed using a Shandon Sequenza staining rack (Thermo Fisher Scientific). Sections were
437 permeabilized for 5 min with 0.1% Triton X-100 and then blocked with a blocking solution (2% BSA
438 plus 0.01% Triton X-100 in PBS) for 1 h at room temperature. The neuron-specific primary antibody
439 against β -III Tubulin (TUJ, mono-clonal mouse anti β -III Tubulin, R&D Systems) was diluted 1:500
440 in blocking solution and incubated overnight at 4°C. The slides were rinsed three times with PBS and
441 incubated with the secondary antibody (Alexa Fluor 488 conjugated, Invitrogen) diluted 1:500 in
442 blocking solution for 2 h at room temperature. After washing three times with PBS, the samples were
443 mounted with a coverslip using Fluoroshield containing DAPI (Sigma). Images of the spiral ganglion
444 were acquired with a Zeiss Axio Imager M1 with Zeiss EC Plan-Neofluar objectives using AxioVision
445 software (AxioVs40 V 4.8.2.0, Carl Zeiss MicroImagin GmbH).

446 **Statistical analysis**

447 Statistical analyses were performed with GraphPad Prism software (Prism 7 for Windows; GraphPad
448 Software Inc., San Diego, CA). If not stated otherwise, results are presented as mean values \pm standard
449 deviations. To compare data between two groups, an unpaired Student t test was used for parametric
450 data. For non-parametric data, a Mann-Whitney test was used. For *in vitro* cytokines and NO release
451 comparing multiple different groups, Tukey's multiple comparison test was applied to adjust for
452 multiple testing. Mortality rates were calculated using log rank (Mantel-Cox) test for significance
453 based on all infected animals and numbers of animals sacrificed for ethical reasons (clinical score of 2)
454 or dying spontaneously. In box-plots, the horizontal line within the box represents the median, the top
455 and bottom of the box mark the 75th and the 25th percentiles, respectively. The upper and lower bound
456 of the whiskers represent the range of the data. A Pearson correlation was performed to correlate
457 cochlear occlusion with hearing capacity. A two-tailed p-value of <0.05 was considered statistically
458 significant, with $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****).

459 For exploratory data analysis, a multivariate linear regression model was used to estimate predictors
460 and determinants for bacterial meningitis-induced hearing loss. The linear coefficients and a 95%

461 confidence interval were calculated for each variable. Statistical analyses were performed using
462 STATA 12 (STATA Corp., College Station, TX).

463 **Acknowledgements**

464 We thank Franziska Simon and Robert Lukesch for excellent technical support. Productive discussions
465 and inputs from the ESCMID Study Group for Infectious Diseases of the Brain (ESGIB) were highly
466 appreciated. This work was supported by a grant from the Swiss National Science Foundation (Grant
467 310030-162583).

468

469 **Conflict of Interests**

470 The authors declare that there are no conflicts of interest regarding the publication of this paper.

471

472 **References**

- 473 1. Koedel U, Scheld WM, Pfister H-W. 2002. Pathogenesis and pathophysiology of
474 pneumococcal meningitis. *Lancet Infect Dis* 2:721–36.
- 475 2. Brouwer MC, Heckenberg SGB, de Gans J, Spanjaard L, Reitsma JB, van de Beek D. 2010.
476 Nationwide implementation of adjunctive dexamethasone therapy for pneumococcal
477 meningitis. *Neurology* 75:1533–1539.
- 478 3. Edmond K, Clark A, Korczak VS, Sanderson C, Griffiths UK, Rudan I. 2010. Global and
479 regional risk of disabling sequelae from bacterial meningitis: a systematic review and meta-
480 analysis. *Lancet Infect Dis* 10:317–328.
- 481 4. Chandran A, Herbert H, Misurski D, Santosham M. 2011. Long-term Sequelae of Childhood
482 Bacterial Meningitis: an underappreciated problem. *Pediatr Infect Dis J* 30:3–6.
- 483 5. Baraff LJ, Lee SI, Schriger DL. 1993. Outcomes of bacterial meningitis in children: a meta-
484 analysis. *Pediatr Infect Dis J* 12:389–94.
- 485 6. van de Beek D, de Gans J, Spanjaard L, Weisfelt M, Reitsma JB, Vermeulen M. 2004. Clinical
486 Features and Prognostic Factors in Adults with Bacterial Meningitis. *N Engl J Med* 351:1849–
487 1859.
- 488 7. McIntyre PB, O'Brien KL, Greenwood B, van de Beek D, Booy R, Heath P. 2012. Effect of
489 vaccines on bacterial meningitis worldwide. *Lancet* (London, England) 380:1703–11.
- 490 8. van de Beek D. 2012. Progress and challenges in bacterial meningitis. *Lancet* 380:1623–1624.
- 491 9. van de Beek D, Farrar JJ, de Gans J, Mai NTH, Molyneux EM, Peltola H, Peto TE, Roine I,
492 Scarborough M, Schultz C, Thwaites GE, Tuan PQ, Zwinderman A. 2010. Adjunctive
493 dexamethasone in bacterial meningitis: a meta-analysis of individual patient data. *Lancet*
494 *Neurol* 9:254–263.
- 495 10. Nau R, Soto A, Bruck W. 1999. Apoptosis of Neurons in the Dentate Gyrus in Humans

- 496 Suffering from Bacterial Meningitis: *J Neuropathol Exp Neurol* 58:265–274.
- 497 11. Gerber J, Raivich G, Wellmer A, Noeske C, Kunst T, Werner A, Brück W, Nau R. 2001. A
498 mouse model of *Streptococcus pneumoniae* meningitis mimicking several features of human
499 disease. *Acta Neuropathol* 101:499–508.
- 500 12. Grandgirard D, Steiner O, Täuber MG, Leib SL. 2007. An infant mouse model of brain damage
501 in pneumococcal meningitis. *Acta Neuropathol* 114:609–617.
- 502 13. Bifrare Y-D, Gianinazzi C, Imboden H, Leib SL, Täuber MG. 2003. Bacterial meningitis
503 causes two distinct forms of cellular damage in the hippocampal dentate gyrus in infant rats.
504 *Hippocampus* 13:481–488.
- 505 14. Grandgirard D, Bifrare Y-D, Pleasure SJ, Kummer J, Leib SL, Täuber MG. 2007.
506 Pneumococcal Meningitis Induces Apoptosis in Recently Postmitotic Immature Neurons in the
507 Dentate Gyrus of Neonatal Rats. *Dev Neurosci* 29:134–142.
- 508 15. Mook-Kanamori BB, Geldhoff M, Poll T van der, Beek D van de. 2011. Pathogenesis and
509 Pathophysiology of Pneumococcal Meningitis. *Clin Microbiol Rev* 24:557–591.
- 510 16. Mitchell L, Smith SH, Braun JS, Herzog K, Weber JR, Tuomanen EI. 2004. Dual Phases of
511 Apoptosis in Pneumococcal Meningitis. *J Infect Dis* 190:2039–2046.
- 512 17. Agyeman P, Grandgirard D, Leib SL. 2014. Chapter 23: Pathogenesis and Pathophysiology of
513 Bacterial Infections., p. . *In* Scheld, MW, Marra, CM, Whitley, RJ (eds.), *Infections of the*
514 *Central Nervous System*. Lippincott Williams & Wilkins.
- 515 18. Iliev AI, Stringaris AK, Nau R, Neumann H. 2003. Neuronal injury mediated via stimulation of
516 microglial toll-like receptor-9 (TLR9). *FASEB J* 18:412–4.
- 517 19. Marques CP, Cheeran MC-J, Palmquist JM, Hu S, Lokensgard JR. 2008. Microglia are the
518 major cellular source of inducible nitric oxide synthase during experimental herpes
519 encephalitis. *J Neurovirol* 14:229–238.

- 520 20. Wellmer A, Noeske C, Gerber J, Munzel U, Nau R. 2000. Spatial memory and learning deficits
521 after experimental pneumococcal meningitis in mice. *Neurosci Lett* 296:137–40.
- 522 21. Loeffler JM, Ringer R, Hablützel M, Täuber MG, Leib SL. 2001. The Free Radical Scavenger
523 α -Phenyl-tert-Butyl Nitron Aggravates Hippocampal Apoptosis and Learning Deficits in
524 Experimental Pneumococcal Meningitis. *J Infect Dis* 183:247–252.
- 525 22. Nau R, Brück W. 2002. Neuronal injury in bacterial meningitis: mechanisms and implications
526 for therapy. *Trends Neurosci* 25:38–45.
- 527 23. Leib SL, Heimgartner C, Bifare Y-D, Loeffler JM, Täuber MG. 2003. Dexamethasone
528 Aggravates Hippocampal Apoptosis and Learning Deficiency in Pneumococcal Meningitis in
529 Infant Rats. *Pediatr Res* 54:353–357.
- 530 24. Perny M, Solyga M, Grandgirard D, Roccio M, Leib SL, Senn P. 2017. Streptococcus
531 pneumoniae -induced ototoxicity in organ of Corti explant cultures. *Hear Res* 350:100–109.
- 532 25. Perny M, Roccio M, Grandgirard D, Solyga M, Senn P, Leib SL. 2016. The Severity of
533 Infection Determines the Localization of Damage and Extent of Sensorineural Hearing Loss in
534 Experimental Pneumococcal Meningitis. *J Neurosci* 36:7740–9.
- 535 26. Klein M, Koedel U, Pfister H-W, Kastenbauer S. 2003. Morphological Correlates of Acute and
536 Permanent Hearing Loss During Experimental Pneumococcal Meningitis. *Brain Pathol* 13:123–
537 132.
- 538 27. Brouwer MC, McIntyre P, Prasad K, van de Beek D. 2015. Corticosteroids for acute bacterial
539 meningitis, p. CD004405. *In* van de Beek, D (ed.), *Cochrane Database of Systematic Reviews*.
540 John Wiley & Sons, Ltd, Chichester, UK.
- 541 28. van de Beek D, Cabellos C, Dzupova O, Esposito S, Klein M, Kloek AT, Leib SL, Mourvillier
542 B, Ostergaard C, Pagliano P, Pfister HW, Read RC, Sipahi OR, Brouwer MC, ESCMID Study
543 Group for Infections of the Brain (ESGIB). 2016. ESCMID guideline: diagnosis and treatment
544 of acute bacterial meningitis. *Clin Microbiol Infect* 22:S37–S62.

- 545 29. Peltola H, Roine I, Fernandez J, Gonzalez Mata A, Zavala I, Gonzalez Ayala S, Arbo A,
546 Bologna R, Goyo J, Lopez E, Mino G, Dourado de Andrade S, Sarna S, Jauhiainen T. 2010.
547 Hearing Impairment in Childhood Bacterial Meningitis Is Little Relieved by Dexamethasone or
548 Glycerol. *Pediatrics* 125:e1–e8.
- 549 30. Spreer A, Gerber J, Hanssen M, Schindler S, Hermann C, Lange P, Eiffert H, Nau R. 2006.
550 Dexamethasone Increases Hippocampal Neuronal Apoptosis in a Rabbit Model of *Escherichia*
551 *coli* Meningitis. *Pediatr Res* 60:210–215.
- 552 31. Coimbra RS, Loquet G, Leib SL. 2007. Limited Efficacy of Adjuvant Therapy with
553 Dexamethasone in Preventing Hearing Loss Due to Experimental Pneumococcal Meningitis in
554 the Infant Rat. *Pediatr Res* 62:291–294.
- 555 32. Grandgirard D, Burri M, Agyeman P, Leib SL. 2012. Adjunctive daptomycin attenuates brain
556 damage and hearing loss more efficiently than rifampin in infant rat pneumococcal meningitis.
557 *Antimicrob Agents Chemother* 56:4289–4295.
- 558 33. Liechti FD, Grandgirard D, Leppert D, Leib SL. 2014. Matrix metalloproteinase inhibition
559 lowers mortality and brain injury in experimental pneumococcal meningitis. *Infect Immun*
560 82:1710–8.
- 561 34. Leib SL, Clements JM, Lindberg RLP, Heimgartner C, Loeffler JM, Pfister L-A, Täuber MG,
562 Leppert D. 2001. Inhibition of matrix metalloproteinases and tumour necrosis factor α
563 converting enzyme as adjuvant therapy in pneumococcal meningitis. *Brain* 124:1734–1742.
- 564 35. Auer M, Pfister LA, Leppert D, Täuber MG, Leib SL. 2000. Effects of clinically used
565 antioxidants in experimental pneumococcal meningitis. *J Infect Dis* 182:347–350.
- 566 36. Högen T, Demel C, Giese A, Angele B, Pfister H-W, Koedel U, Klein M. 2013. Adjunctive N-
567 acetyl-L-cysteine in treatment of murine pneumococcal meningitis. *Antimicrob Agents*
568 *Chemother* 57:4825–30.
- 569 37. Klein M, Koedel U, Pfister H-W, Kastenbauer S. 2003. Meningitis-associated hearing loss:

- 570 Protection by adjunctive antioxidant therapy. *Ann Neurol* 54:451–458.
- 571 38. Masouris I, Klein M, Dyckhoff S, Angele B, Pfister HW, Koedel U. 2017. Inhibition of DAMP
572 signaling as an effective adjunctive treatment strategy in pneumococcal meningitis. *J*
573 *Neuroinflammation* 14:214.
- 574 39. Woehrl B, Brouwer MC, Murr C, Heckenberg SGB, Baas F, Pfister HW, Zwinderman AH,
575 Morgan BP, Barnum SR, van der Ende A, Koedel U, van de Beek D. 2011. Complement
576 component 5 contributes to poor disease outcome in humans and mice with pneumococcal
577 meningitis. *J Clin Invest* 121:3943–53.
- 578 40. Mook-Kanamori BB, Rouse MS, Kang C-I, van de Beek D, Steckelberg JM, Patel R. 2009.
579 Daptomycin in experimental murine pneumococcal meningitis. *BMC Infect Dis* 9:50.
- 580 41. Kasanmoentalib ES, Valls Seron M, Morgan BP, Brouwer MC, van de Beek D. 2015.
581 Adjuvant treatment with dexamethasone plus anti-C5 antibodies improves outcome of
582 experimental pneumococcal meningitis: a randomized controlled trial. *J Neuroinflammation*
583 12:149.
- 584 42. Muri L, Grandgirard D, Buri M, Perny M, Leib SL. 2018. Combined effect of non-bacteriolytic
585 antibiotic and inhibition of matrix metalloproteinases prevents brain injury and preserves
586 learning, memory and hearing function in experimental paediatric pneumococcal meningitis. *J*
587 *Neuroinflammation* 15:233.
- 588 43. Klein M, Höhne C, Angele B, Högen T, Pfister HW, Tüfekci H, Koedel U. 2018. Adjuvant
589 non-bacteriolytic and anti-inflammatory combination therapy in pneumococcal meningitis: an
590 investigation in a mouse model. *Clin Microbiol Infect*.
- 591 44. Stucki A, Cottagnoud M, Winkelmann V, Schaffner T, Cottagnoud P. 2007. Daptomycin
592 Produces an Enhanced Bactericidal Activity Compared to Ceftriaxone, Measured by
593 [3H]Choline Release in the Cerebrospinal Fluid, in Experimental Meningitis Due to a
594 Penicillin-Resistant Pneumococcal Strain without Lysing Its Cell Wall. *Antimicrob Agents*

- 595 Chemother 51:2249–2252.
- 596 45. Grandgirard D, Schürch C, Cottagnoud P, Leib SL. 2007. Prevention of brain injury by the
597 nonbacteriolytic antibiotic daptomycin in experimental pneumococcal meningitis. *Antimicrob*
598 *Agents Chemother* 51:2173–8.
- 599 46. Gerber P, Stucki A, Acosta F, Cottagnoud M, Cottagnoud P. 2006. Daptomycin is more
600 efficacious than vancomycin against a methicillin-susceptible *Staphylococcus aureus* in
601 experimental meningitis. *J Antimicrob Chemother* 57:720–723.
- 602 47. Kullar R, Chin JN, Edwards DJ, Parker D, Coplin WM, Rybak MJ. 2011. Pharmacokinetics of
603 single-dose daptomycin in patients with suspected or confirmed neurological infections.
604 *Antimicrob Agents Chemother* 55:3505–9.
- 605 48. Cottagnoud P, Pfister M, Acosta F, Cottagnoud M, Flatz L, Kuhn F, Muller H-P, Stucki A.
606 2004. Daptomycin Is Highly Efficacious against Penicillin-Resistant and Penicillin- and
607 Quinolone-Resistant *Pneumococci* in Experimental Meningitis. *Antimicrob Agents Chemother*
608 48:3928–3933.
- 609 49. Yim CW, Flynn NM, Fitzgerald FT. 1985. Penetration of oral doxycycline into the
610 cerebrospinal fluid of patients with latent or neurosyphilis. *Antimicrob Agents Chemother*
611 28:347–8.
- 612 50. Brown DL, Desai KK, Vakili BA, Nouneh C, Lee H-M, Golub LM. 2004. Clinical and
613 Biochemical Results of the Metalloproteinase Inhibition with Subantimicrobial Doses of
614 Doxycycline to Prevent Acute Coronary Syndromes (MIDAS) Pilot Trial. *Arterioscler Thromb*
615 *Vasc Biol* 24:733–738.
- 616 51. Roach D., Fitridge R., Laws P., Millard S., Varelias A, Cowled P. 2002. Up-regulation of
617 MMP-2 and MMP-9 Leads to Degradation of Type IV Collagen During Skeletal Muscle
618 Reperfusion Injury; Protection by the MMP Inhibitor, Doxycycline. *Eur J Vasc Endovasc Surg*
619 23:260–269.

- 620 52. Golub LM, Lee H-M, Ryan ME, Giannobile WV, Payne J, Sorsa T. 1998. Tetracyclines Inhibit
621 Connective Tissue Breakdown by Multiple Non-Antimicrobial Mechanisms. *Adv Dent Res*
622 12:12–26.
- 623 53. Pasquale TR, Tan JS. 2005. Nonantimicrobial Effects of Antibacterial Agents. *Clin Infect Dis*
624 40:127–135.
- 625 54. Gabler WL, Creamer HR. 1991. Suppression of human neutrophil functions by tetracyclines. *J*
626 *Periodontal Res* 26:52–8.
- 627 55. Suomalainen K, Sorsa T, Golub LM, Ramamurthy N, Lee HM, Uitto VJ, Saari H, Kontinen
628 YT. 1992. Specificity of the anticollagenase action of tetracyclines: relevance to their anti-
629 inflammatory potential. *Antimicrob Agents Chemother* 36:227–9.
- 630 56. Meli DN, Coimbra RS, Erhart DG, Loquet G, Bellac CL, Täuber MG, Neumann U, Leib SL.
631 2006. Doxycycline Reduces Mortality and Injury to the Brain and Cochlea in Experimental
632 Pneumococcal Meningitis. *Infect Immun* 74:3890–3896.
- 633 57. Paul R, Lorenzl S, Koedel U, Sporer B, Vogel U, Frosch M, Pfister H-W. 1998. Matrix
634 metalloproteinases contribute to the blood-brain barrier disruption during bacterial meningitis.
635 *Ann Neurol* 44:592–600.
- 636 58. Leib SL, Leppert D, Clements J, Täuber MG. 2000. Matrix metalloproteinases contribute to
637 brain damage in experimental pneumococcal meningitis. *Infect Immun* 68:615–20.
- 638 59. The European Committee on Antimicrobial Susceptibility Testing. 2019. Breakpoint tables for
639 interpretation of MICs and zone diameters, version 9.0.
- 640 60. Pankuch GA, Jacobs MR, Appelbaum PC. 2003. Bactericidal activity of daptomycin against
641 *Streptococcus pneumoniae* compared with eight other antimicrobials. *J Antimicrob Chemother*
642 51:443–6.
- 643 61. Tomasz A, Moreillon P, Pozzi G. 1988. Insertional inactivation of the major autolysin gene of
644 *Streptococcus pneumoniae*. *J Bacteriol* 170:5931–4.

- 645 62. Tuomanen E, Liu H, Hengstler B, Zak O, Tomasz A. 1985. The induction of meningeal
646 inflammation by components of the pneumococcal cell wall. *J Infect Dis* 151:859–68.
- 647 63. Nau R, Eiffert H. 2005. Minimizing the release of proinflammatory and toxic bacterial products
648 within the host: A promising approach to improve outcome in life-threatening infections.
649 *FEMS Immunol Med Microbiol* 44:1–16.
- 650 64. Nau R, Eiffert H. 2002. Modulation of release of proinflammatory bacterial compounds by
651 antibacterials: potential impact on course of inflammation and outcome in sepsis and
652 meningitis. *Clin Microbiol Rev* 15:95–110.
- 653 65. Leppert D, Leib SL, Grygar C, Miller KM, Schaad UB, Hollander GA. 2000. Matrix
654 Metalloproteinase (MMP)-8 and MMP-9 in Cerebrospinal Fluid during Bacterial Meningitis:
655 Association with Blood-Brain Barrier Damage and Neurological Sequelae. *Clin Infect Dis*
656 31:80–84.
- 657 66. Rosenberg GA. 2002. Matrix metalloproteinases in neuroinflammation. *Glia* 39:279–291.
- 658 67. Yang Y, Estrada EY, Thompson JF, Liu W, Rosenberg GA. 2007. Matrix Metalloproteinase-
659 Mediated Disruption of Tight Junction Proteins in Cerebral Vessels is Reversed by Synthetic
660 Matrix Metalloproteinase Inhibitor in Focal Ischemia in Rat. *J Cereb Blood Flow Metab*
661 27:697–709.
- 662 68. McColl BW, Rothwell NJ, Allan SM. 2008. Systemic Inflammation Alters the Kinetics of
663 Cerebrovascular Tight Junction Disruption after Experimental Stroke in Mice. *J Neurosci*
664 28:9451–9462.
- 665 69. Sellner J, Leib SL. 2006. In bacterial meningitis cortical brain damage is associated with
666 changes in parenchymal MMP-9/TIMP-1 ratio and increased collagen type IV degradation.
667 *Neurobiol Dis* 21:647–656.
- 668 70. Leppert D, Lindberg RLP, Kappos L, Leib SL. 2001. Matrix metalloproteinases:
669 multifunctional effectors of inflammation in multiple sclerosis and bacterial meningitis. *Brain*

- 670 Res Rev 36:249–257.
- 671 71. Khokha R, Murthy A, Weiss A. 2013. Metalloproteinases and their natural inhibitors in
672 inflammation and immunity. *Nat Rev Immunol* 13:649–665.
- 673 72. Song J, Wu C, Zhang X, Sorokin LM. 2013. In Vivo Processing of CXCL5 (LIX) by Matrix
674 Metalloproteinase (MMP)-2 and MMP-9 Promotes Early Neutrophil Recruitment in IL-1 -
675 Induced Peritonitis. *J Immunol* 190:401–410.
- 676 73. Lee CZ, Xu B, Hashimoto T, McCulloch CE, Yang G-Y, Young WL. 2004. Doxycycline
677 Suppresses Cerebral Matrix Metalloproteinase-9 and Angiogenesis Induced by Focal
678 Hyperstimulation of Vascular Endothelial Growth Factor in a Mouse Model. *Stroke* 35:1715–
679 1719.
- 680 74. Clark WM, Lessov N, Lauten JD, Hazel K. 1997. Doxycycline treatment reduces ischemic
681 brain damage in transient middle cerebral artery occlusion in the rat. *J Mol Neurosci* 9:103–
682 108.
- 683 75. Steenbergen JN, Alder J, Thorne GM, Tally FP. 2005. Daptomycin: a lipopeptide antibiotic for
684 the treatment of serious Gram-positive infections. *J Antimicrob Chemother* 55:283–288.
- 685 76. Sauermann R, Rothenburger M, Graninger W, Joukhadar C. 2008. Daptomycin: A Review 4
686 Years after First Approval. *Pharmacology* 81:79–91.
- 687 77. Baltz RH. 2009. Daptomycin: mechanisms of action and resistance, and biosynthetic
688 engineering. *Curr Opin Chem Biol* 13:144–151.
- 689 78. Grandgirard D, Oberson K, Bühlmann A, Gäumann R, Leib SL. 2010. Attenuation of
690 cerebrospinal fluid inflammation by the nonbacteriolytic antibiotic daptomycin versus that by
691 ceftriaxone in experimental pneumococcal meningitis. *Antimicrob Agents Chemother*
692 54:1323–1326.
- 693 79. Barichello T, Gonçalves JCN, Generoso JS, Milioli GL, Silvestre C, Costa CS, Coelho J da R,
694 Comim CM, Quevedo J. 2013. Attenuation of cognitive impairment by the nonbacteriolytic

- antibiotic daptomycin in Wistar rats submitted to pneumococcal meningitis. *BMC Neurosci* 14:42.
80. Maitra SR, Bhaduri S, Valane PD, Tervahartiala T, Sorsa T, Ramamurthy N. 2003. Inhibition of matrix metalloproteinases by chemically modified tetracyclines in sepsis. *Shock* 20:280–5.
81. Solomon A, Rosenblatt M, Li DQ, Liu Z, Monroy D, Ji Z, Lokeshwar BL, Pflugfelder SC. 2000. Doxycycline inhibition of interleukin-1 in the corneal epithelium. *Invest Ophthalmol Vis Sci* 41:2544–57.
82. Senn P, Rostetter C, Arnold A, Kompis M, Vischer M, Häusler R, Ozdoba C, Mantokoudis G, Caversaccio M. 2012. Retrograde cochlear implantation in postmeningitic basal turn ossification. *Laryngoscope* 122:2043–2050.
83. Bally L, Grandgirard D, Leib SL. 2016. Inhibition of Hippocampal Regeneration by Adjuvant Dexamethasone in Experimental Infant Rat Pneumococcal Meningitis. *Antimicrob Agents Chemother* 60:1841–6.
84. Zysk G, Brück W, Gerber J, Brück Y, Prange HW, Nau R. 1996. Anti-inflammatory treatment influences neuronal apoptotic cell death in the dentate gyrus in experimental pneumococcal meningitis. *J Neuropathol Exp Neurol* 55:722–8.
85. Zwijnenburg PJG, van der Poll T, Florquin S, van Deventer SJH, Roord JJ, van Furth AM. 2001. Experimental Pneumococcal Meningitis in Mice: A Model of Intranasal Infection. *J Infect Dis* 183:1143–1146.
86. Piva S, Di Paolo A, Galeotti L, Ceccherini F, Cordoni F, Signorini L, Togni T, De Nicolò A, Rasulo FA, Fagoni N, Latronico N, D’Avolio A. 2019. Daptomycin Plasma and CSF Levels in Patients with Healthcare-Associated Meningitis. *Neurocrit Care*.
87. Hupp S, Heimeroth V, Wippel C, Förtsch C, Ma J, Mitchell TJ, Iliev AI. 2012. Astrocytic tissue remodeling by the meningitis neurotoxin pneumolysin facilitates pathogen tissue penetration and produces interstitial brain edema. *Glia* 60:137–146.

- 720 88. Iliev AI, Stringaris AK, Nau R, Neumann H. 2004. Neuronal injury mediated via stimulation of
721 microglial toll-like receptor-9 (TLR9). *FASEB J* 18:412–414.
- 722 89. Klugman KP, Friedland IR, Bradley JS. 1995. Bactericidal activity against cephalosporin-
723 resistant *Streptococcus pneumoniae* in cerebrospinal fluid of children with acute bacterial
724 meningitis. *Antimicrob Agents Chemother* 39:1988–1992.
- 725 90. Karlsson M, Hammers S, Nilsson-Ehle I, Malmberg AS, Wretling B. 1996. Concentrations of
726 doxycycline and penicillin G in sera and cerebrospinal fluid of patients treated for
727 neuroborreliosis. *Antimicrob Agents Chemother* 40:1104–7.
- 728 91. Gianinazzi C, Grandgirard D, Imboden H, Egger L, Meli DN, Bifare Y-D, Joss PC, Täuber
729 MG, Borner C, Leib SL. 2003. Caspase-3 mediates hippocampal apoptosis in pneumococcal
730 meningitis. *Acta Neuropathol* 105:499–507.
- 731 92. Gehre F, Leib SL, Grandgirard D, Kummer J, Bhlmann A, Simon F, Gumann R, Kharat AS,
732 Tuber MG, Tomasz A. 2008. Essential role of choline for pneumococcal virulence in an
733 experimental model of meningitis. *J Intern Med* 264:143–154.
- 734 93. van Furth AM, Roord JJ, van Furth R. 1996. Roles of proinflammatory and anti-inflammatory
735 cytokines in pathophysiology of bacterial meningitis and effect of adjunctive therapy. *Infect*
736 *Immun* 64:4883–90.

737

738

739 **Figure legends**

740 **Fig. 1 Inflammatory cytokines and nitric oxide (NO) production upon *in vitro* stimulation of**
741 **astroglial cells with living *S. pneumoniae*.** Cytokines and NO were measured in cell culture
742 supernatant 24 hours after concomitant application of living *S. pneumoniae* and antibiotics. Infection
743 and bacteriolysis induced by ceftriaxone (CRO) resulted in high levels of IL-1 β (**A**), IL-6 (**B**), TNF- α
744 (**D**) and NO (**F**) release, whereas no clear effect was found for IFN- γ (**E**). All therapies containing
745 non-bacteriolytic antibiotics significantly reduced the release of inflammatory cytokines compared to
746 CRO monotherapy (all $p < 0.01$ for CRO vs. DOX; $p < 0.001$ for CRO vs. CRO+DOX, CRO vs. DAP,
747 CRO vs. CRO+DAP; $p < 0.0001$ for CRO vs. CRO+DOX+DAP). Doxycycline (DOX) monotherapy
748 and all combination therapies containing daptomycin (DAP) significantly reduced NO production
749 compared to CRO monotherapy (all $p < 0.0001$). Significance levels are not indicated in the graphs for
750 clearer representation. Statistical differences were assessed using unpaired t-tests.

751

752

753 **Fig.2 Clinical data of infant rats during acute pneumococcal meningitis.** Combined antibiotic
754 therapy (n=72) with daptomycin (DAP), doxycycline (DOX) and ceftriaxone (CRO) significantly
755 improved survival compared to CRO monotherapy (n=71) during acute PM (**A**). Relative weight
756 change during acute PM indicates reduced weight gain in infected (PM+) compared to mock-infected
757 (PM-) animals (n=6 per treatment group). Infected animals with combined adjuvant therapy presented
758 significantly increased weight change at 42 hpi, compared to CRO monotherapy (**B**). Clinical scoring
759 revealed reduced clinical scores in all infected compared to mock-infected animals (**C**). Differences
760 were assessed by an unpaired t-test in (B) and (C) and with a Log-rank test to compare survival in A.

761 **Fig. 3 Inflammatory CSF cytokine levels, cortical necrosis and bacterial clearance from CSF**
762 **during the acute PM.** Cytokine levels are represented by mean \pm 95% confidence interval starting
763 before treatment initiation, 6 hours and 24 hours after treatment start (representing 18, 24 and 42 hpi)
764 for IL-1 β (A), IL-6 (B), IL-10 (C) and TNF- α (D). Combination adjuvant therapy (n=11) significantly
765 reduced IL-1 β , IL-6 and IL-10 CSF levels compared to CRO monotherapy (n=11), while showing a
766 trend towards reduced TNF- α CSF level 6 hours after treatment start. Bacterial titers in the CSF were
767 similar in both treatment groups before starting therapy (at 18 hpi; n=53 for CRO/DAP/DOX, n=49 for
768 CRO) with a faster bacterial clearance in animals receiving the combined adjuvant therapy (n=17)
769 compared to CRO monotherapy (n=18, E). Cortical necrosis was only found in animals with PM and
770 was significantly reduced by combined adjuvant therapy (n=15) compared to CRO monotherapy
771 (n=15, F). Statistical differences were assessed using an unpaired t-test for cytokines and bacterial titer
772 at 18 hpi. For necrotic cortex volume and bacterial titer at 24 hpi a Mann-Whitney test was used as
773 data were not normally distributed.

774

775 **Fig. 4 Hearing capacity assessed three weeks after acute pneumococcal meningitis.**

776 Representative ABR recordings from a mock-infected rat (**A**) with a hearing threshold of 35dB
777 compared to an infected rat (**B**) with an elevated hearing threshold of 65dB. Perceived sound
778 intensities are indicated with dashed lines. Infected animals receiving combined adjuvant therapy
779 (n=25) showed a trend towards improved hearing thresholds compared to animals receiving CRO
780 monotherapy (n=23, **C**). Combined adjuvant therapy significantly reduced hearing loss in mildly
781 infected animals (n=10) compared to CRO monotherapy (n=9, **D**).

782

783 **Fig. 5 Immunohistological analysis of spiral ganglion neuron density and cochlear occlusion in**
784 **mildly infected animals. (A)** Midmodiolar section showing the basal (B), middle (M) and apical (A)
785 turns of an infected rat three weeks after PM immunostained for neurons (β -III tubulin, green) and cell
786 nuclei (DAPI, blue). The absence and presence of fibrous occlusion in the perilymphatic space in a
787 mock-infected control animal and in an infected animal receiving standard CRO monotherapy is
788 represented in over-exposed immunofluorescence pictures (**B**). The area with fibrous occlusion in the
789 perilymphatic space is indicated by white dashed lines. Spiral ganglion neuron (SGN) density was not
790 significantly different in any of the cochlear turns in infected animals between the two treatment
791 groups (**C**). Combined adjuvant therapy (n=10) significantly reduced the amount of fibrous tissue in
792 the perilymphatic space compared to CRO monotherapy (n=9, **D**). A significant positive correlation
793 for fibrous occlusion of the perilymphatic space and hearing threshold was found (**E**).

794

795

796 **Table with legend**

797 **Table 1.** Univariate and multivariate linear regression for click hearing thresholds three weeks after
 798 surviving an episode of acute pneumococcal meningitis (n=48).

Hearing Thresholds						
	Univariate			Multivariate		
	Coef.	95% CI	p	Coef.	95% CI	p
Bacterial inoculum (per add. log)	70.5	45.6-95.5	<0.001	69.9	48.2-91.6	<0.001
Comb. Adjuv. Therapy	-9.7	-23.8-4.4	0.172	-10.3	-19.9- -0.6	0.037
Low in-litter mortality	-18.1	-31.8- -4.3	0.011	-17.1	-26.9- -7.3	0.001
Activity @ 24hpi (per add. score)	-26.7	-39.5- -14.02	<0.001	-	-	-

799









